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(54) Title: VON WILLEBRAND FACTOR (vWF) CLEAVING PROTEASE POLYPEPTIDE, NUCLEIC ACID ENCODING THE
POLYPEPTIDE AND USE OF POLYPEPTIDE

(57) Abstract: The invention relates to a vWF cleaving protease (vWF-cp) polypeptide, a nucleic acid molecule encoding the amino acid sequence of a vWF-cp polypeptide and a composition comprising the polypeptide. The invention also relates to the use of the vWF cleaving protease polypeptide for production of vWF cleaving protease polypeptide binding molecules and for production of a preparation for prophylaxis and therapy of thrombosis and thromboembolic disease.

**von Willebrand Factor (vWF) cleaving protease polypeptide,
nucleic acid encoding the polypeptide and use of
polypeptide**

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Field of the Invention

The invention relates to a vWF cleaving protease (vWF-cp) polypeptide or a partial sequence thereof, a nucleic acid molecule encoding the amino acid sequence of a vWF -cp polypeptide, and a composition comprising the polypeptide.

10 The invention also relates to the use of the vWF-cp polypeptide for production of anti-vWF-cp polypeptide antibodies and for production of a preparation for prophylaxis and therapy of thrombosis and thromboembolic disease.

15 **Background of the Invention**

vWF is a glycoprotein circulating in plasma as a series of multimers ranging in size from about 500 to 20,000 kD. Multimeric forms of vWF are composed of 250 kD polypeptide subunits linked together by disulfide bonds.

20 vWF mediates the initial platelet adhesion to the subendothelium of a damaged vessel wall, though only the largest multimers appear to exhibit haemostatic activity. Such vWF multimers having large molecular masses are stored in the Weibel Palade bodies of endothelial cells, and it is believed that endothelial cells secrete these large 25 polymeric forms of vWF. Those forms of vWF which have a low molecular weight (low molecular weight or LMW vWF) are believed to arise from proteolytic cleavage of the larger multimers.

30 A small portion of the vWF present in normal plasma circulates as 189, 176 and 140 kD fragments resulting from

proteolytic degradation of vWF *in vivo*, the 140 kD fragment being derived from the N-terminal region, and the 176 kD fragment from the C-terminal region of the subunit. When low molecular weight (LMW) forms of vWF are isolated from 5 normal human plasma and subjected to SDS-PAGE (polyacrylamide gel electrophoreses) after disulfide reduction, an unusually high portion of vWF fragments are found. This finding is compatible with the view that LMW forms of vWF have been partially or predominantly derived 10 from large multimers by proteolytic degradation.

The proteolytic degradation of vWF is a physiological process in healthy individuals, yet in patients suffering from von Willebrand disease (vWD) type 2A it may be accelerated, and as a consequence these patients lack the 15 vWF multimers with the largest molecular masses. A lack of large vWF multimers and an increased level of proteolytic fragments are also observed in acquired von Willebrand disease (vWD) associated with myeloproliferation syndrome, indicating increased *in vivo* proteolysis in this condition 20 as well.

In patients with thrombotic thrombocytopenic purpura (TTP), on the other hand, unusually large vWF multimers are detected, and increased vWF binding to platelets has been demonstrated in these patients (Moake et al. 1982, N. Engl. 25 J. Med. 307: 1432-1435). Familial TTP is associated with a severe congenital deficiency of vWF protease, while the presence of vWF-cleaving proteases inhibiting autoantibodies has been observed in patients with non-familial TTP.

30 The large multimers of vWF associated with TTP normally disappear after a patient is transfused with normal fresh frozen plasma. Presently, plasma exchange is

the most important treatment for TTP, although significant side effects have been reported with this therapy. The existence of a severe congenital deficiency of vWF protease has been established in patients with familial TTP and the 5 presence of a vWF-cleaving protease inhibiting autoantibodies has been observed in patients with non-familial TTP.

Several proteases have been shown to be able to cleave vWF, thereby impairing its binding affinity for platelets. 10 However, *in vitro* the cleavage of vWF with these proteases in each case results in cleavage products different from the fragments derived from *in vivo* cleavage.

Thus, for example, while plasmin is capable of cleaving several peptide bonds in vWF, plasmin-treated vWF 15 retains a high molecular weight core region retaining about 70% of its platelet agglutinating activity (determined as ristocetin cofactor). A 34 kD peptide is split from the N-termini of individual vWF subunits in the early stages of plasmin treatment, and epitope mapping of such plasmin-20 induced fragments show that these fragments originated from regions of the vWF subunit that are different from the vWF fragments present in circulating plasma.

Porcine pancreatic elastase and various serine proteases released from human leukocytes have also been 25 shown to degrade vWF proteolytically with a resultant loss of large multimers. Epitope mapping of the degradation products again indicates that these fragments also differ from those present in normal plasma and in vWD type 2A. In addition, a calpain-like protease released from human 30 platelets has been shown to degrade large vWF multimers and to create vWF fragments similar to those observed *in vivo*.

Summary of the Invention

It is an object of the invention to provide for a vWF cleaving protease (vWF-cp) polypeptide or a partial sequence thereof.

5 It is another object of the invention to provide for a composition comprising a vWF-cp polypeptide.

It is an object of the present invention to provide for a nucleic acid molecule comprising a nucleic acid sequence encoding an amino acid sequence of a vWF-cp polypeptide.

10 It is also an object of the invention to provide for recombinant vWF-cp polypeptide.

It is another object of the invention to provide a method of production of a recombinant vWF-cp polypeptide.

15 It is also an object of the invention to provide for a method of purification of vWF using a vWF-cp polypeptide or a partial sequence thereof.

It is also an object of the invention to provide for anti-vWF-cp polypeptide ligands.

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Brief Description of the Drawing

Fig. 1 shows the schematic purification scheme of the vWF-cp from plasma.

25 Fig. 2 shows the SDS-PAGE of purified vWF-cp polypeptides under non-reducing (A) and reducing conditions in the presence of DTT (B).

Fig. 3 shows the partial nucleotide and amino acid sequence of the vWF-cp polypeptide.

30 Fig. 4 shows the schematic drawing of the genomic localization of the vWF-cleaving protease gene.

Fig. 5 shows the complete amino acid and nucleotide sequence of the vWF-cleaving protease. Start and end of the

signal peptide, metalloprotease, the cystein rich region (ACR), thromspondin type 1 motif (TSP 1), and disintegrin like motif (RGD) domains are indicated by arrows. Furin cleavage site, catalytic site and Met-turn are underlined.

5 The putative N-glycosylation sites are indicated by asterisks.

Fig. 6 shows the schematic representation of the domain organization of the vWF-cleaving protease and those of the human members of the ADAM-TS family. The domain 10 structures of the vWF-cleaving protease (ADAMTS 13) and of all known human ADAMTS proteins are shown. ADAMTS 10 cannot be found in databases and ADAMTS 11 is identical to ADAMTS 5.

Fig. 7 shows Western blot analysis of cells 15 transfected with a vector comprising vWF-cp polypeptide encoding sequences, with lane 1: standard molecular weight marker, lane 2: control vector cDNA3.1(+) and lane 3: vector pCMV-vWFcp. The specific vWF-cp polypeptide band is indicated by the arrow.

Fig. 8 shows SDS-Page of vWF-cp activity on vWF with 20 lane 1: purified plasmatic vWF as control, lane 2: vWF incubated with normal human plasma, lane 3: vWF incubated with buffer as control, lane 4: vWF incubated with of cell lysate of SK-Hep cells transfected with control vector cDNA3.1(+) and lane 5: vWF incubated with of cell lysate of 25 SK Hep cells transfected with vWF-cp expressing vector pCMV-vWFcp.

Detailed Description of the Invention

30 In accordance with one of the objects of the invention there is provided a vWF-cp polypeptide comprising (a) an amino acid sequence selected from the group of SEQ ID. NO.

1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4 and SEQ ID NO. 5, or (b) an amino acid sequence having at least 80% identity to the amino acid sequence of (a). Polypeptide molecules can also have at least 90%, or at least 95% 5 identity to the amino acid sequence of (a). The polypeptide preferably has an amino acid sequence as shown in Fig. 5 or a partial sequence thereof of at least 12, preferably at least 15 amino acids. Preferably, the vWF-cp polypeptide has vWF cleaving activity. The vWF-cp polypeptide of the 10 invention preferably retains vWF protease activity in the presence of serine protease inhibitor and a calpain protease inhibitor. The serine protease inhibitor can be diisopropyl fluorophosphate or any analogue thereof which has serine protease inhibitor activity. The calpain 15 inhibitor can be Z-Leu-Leu-Tyr-CHN2 or any analogue thereof having calpain inhibitor activity.

The term "polypeptide" means a chain of amino acid residues linked through peptide bonds between the α -carboxyl carbon of one amino acid residue and the α -nitrogen of the next amino acid, and comprising 10 or more amino acid residues joint together. The polypeptide may 20 contain other than the 20 gene-encoded amino acids. "Polypeptide" refers both to short chains, such as 25 peptides, oligopeptides, or oligomers, and to longer chains, generally referred to as proteins. "Polypeptide" include amino acid sequences modified either by natural processes, such as posttranslational modifications, or chemical modifications, which are known in the art. 30 Modifications can occur anywhere in the polypeptide. The modification may lead to a variant of the polypeptide which

may differ in amino acid sequence from original polypeptide by one or more substitutions, additions, deletions, fusions or any combination, or a naturally occurring variant, such as an allelic variant or non-naturally occurring variant
5 made by mutagenesis or genetic engineering. The "Polypeptide" may be in form of an unprocessed or partial processed precursor, the "mature" polypeptide, or a fragment having an amino acid sequence that entirely is the same as a part, but not all, of the amino acid sequence of
10 the longer a polypeptide chain. Fragment may be a shorter single continuous region of the longer chain, or comprised within a larger polypeptide of which they form a part. A Fragment of polypeptide may include, for example a truncated polypeptide having a partial amino acid sequence
15 of the vWF-cp. A "Fragment" can be a biological active fragment that mediate vWF cleaving protease activity, including those with similar activity or improved activity, or with decreased activity.

"Identity" means the identity of the amino acid sequence of a polypeptide to the amino acid sequence of a polypeptide comprising the sequence of SEQ ID. NO 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4 or SEQ ID NO. 5. "At least 80% identity", means that the amino acid sequence is identical except that the polypeptide sequence differs in not less than 20 amino acids per 100 amino acids. "At least 90%" or "at least 95% identity" means a difference of not less than 10, or not less than 5 amino acids per 100 amino acids.

30 The term "vWF cleaving protease" (vWF-cp) means a protein or polypeptide having vWF cleaving activity and cleaves vWF high molecular vWF multimers in molecules of

lower molecular weight which still have vWF activity and properties.

The term "vWF cleaving protease polypeptide" means a polypeptide having at least 10 amino acid residues. The 5 complete amino acid sequence of the unprocessed polypeptide comprises 1427 amino acids. The "mature" polypeptide, which is a part of the larger unprocessed polypeptide should normally begin with or near amino acid 75, beginning with AAGGILH, and continuing to the carboxyl terminus. The 10 mature vWF cleaving protease has a calculated polypeptide mass of about 145 KD. Additional amino acids which contains secretory or leader sequences, pro-sequences, sequences which aid purification or identification such as multiple His residues, a FLAG tag or and additional sequence for 15 stability during recombinant production, can be included.

The term "vWF cleaving activity" means a physiological vWF cleaving activity which is defined by (1) the cleaving vWF at the peptide bond 842Tyr-843Met, (2) having a direct proteolytic activity which converts vWF having a singlet 20 structure to vWF having a satellite structure, and (3) retaining activity in the presence of a serine protease inhibitor such as diisopropyl fluorophosphate (DFP) and in the presence of a calpain protease inhibitor such as carbobenzyloxy (Z) peptidyl diazomethylketone inhibitor (Z- 25 Leu-Leu-Tyr-CHN₂). The proteolytic entities provided with the present invention may also act indirectly via another effector protein, for example a protease. This polypeptide can form an active vWF cleaving complex together with a metal ion selected from the group consisting Ca⁺⁺, Sr⁺⁺, Mg⁺⁺ and Ba⁺⁺. The preferred metal ion is Ca⁺⁺. This active 30 complex is able to cleave vWF in a physiological manner as

described above. The vWF cleaving activity of the polypeptide according to the present invention may be determined by any method described in the art, such as the method according to Furlan et al. (1996, Blood 87: 4223-4234) or as described in WO 00/50904. Alternatively, a collagen binding assay, e.g. as described in EP 0 816 852, can also be used as a test system.

According to one aspect, the invention provides for an isolated polypeptide comprising an amino acid sequence having at least 80% identity to the amino acid sequence selected from the group of SEQ ID. NO 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4 and SEQ ID NO. 5. According to one embodiment of this aspect of the invention the isolated polypeptide comprises an amino acid sequence selected from the group of SEQ ID. NO 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4 and SEQ ID NO. 5.

The term "isolated" means altered from the natural state and/or removed from its original environment.

The invention also provides for a substantially pure vWF-cp polypeptide comprising (a) an amino acid sequence of selected from the group of SEQ ID. NO 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4 and SEQ ID NO. 5, or (b) an amino acid sequence having at least 80% identity to the amino acid sequence of (a).

The term "substantially pure" means purity as high as the relative proportions of polypeptide chains with the vWF-cp activity present in an amount of above 50%, especially above 80%, most preferred about 90%, of total protein compared to the vWF protease activity in plasma. The purity is determined by a purity as determined by SDS-

PAGE (silver stained or Commassie stained) and Western blot and a ratio of vWF protease polypeptide to total protein amount, said purity is preferably greater than about 98%. If the purified vWF-cp polypeptide is purified from plasma 5 the preparation contains between 0.001% and 1%, preferably 0.002% of the initial amount of plasma protein, and at least 1%, preferably at least 2.3% of the initial enzyme activity, which may be partially inactivated during the purification procedure. The preparation comprising a vWF - 10 cp polypeptide according to the present invention is essentially free of vWF or vWF fragments, i.e. having a vWF content of below 5%, preferably below the detection limit of an assay used to detect vWF, such as a collagen-assay described in EP 0 816 852.

15 The substantially pure vWF-cp polypeptide exhibits an apparent molecular weight of about 180 KD, 170 KD, 160 KD or 120 KD in SDS-PAGE analysis under reducing conditions.

20 The substantially pure vWF protease polypeptide exhibits an apparent molecular weight of about 150 KD, 140 KD, 130 KD or 110 KD in SDS-PAGE analysis under non-reducing conditions.

25 The SDS-PAGE is performed under reducing conditions or non-reducing conditions. It is well known in the art, that molecular weight determination using SDS PAGE results in the detection of apparent molecular masses, which may be different from the molecular masses of the native, non-denatured protein.

30 Analysis of non-denatured material by mass spectrometry showed very broad peaks of high molecular weight. This finding is in agreement with appearance, in gel filtration experiments, suggesting that the proteins in this preparation tend to polymerize under physiologic

conditions (a property of clusterin).

The polypeptide of the invention can be isolated from any source which comprising a vWF-cp polypeptide.

5 The term "source" means human plasma, a supernatant of a cell culture expressing a vWF -cp polypeptide according to the present invention, milk or other body fluids of transgenic animals expressing the polypeptide of the invention.

10 Purification of vWF-cp polypeptide can be performed by a combination of chromatographic steps including immunoaffinity chromatography, gel filtration, and ion exchange chromatography. For example, the first purification step can be immunoaffinity chromatography, the 15 second step can be gel filtration, followed by one or more additional immunoaffinity chromatography steps. A further purification can be performed using ion exchange chromatography, preferably anion exchange chromatography and at least one affinity chromatography. Further 20 purification steps can be performed using ion exchange chromatography, gel filtration and further affinity chromatography steps.

25 According to one aspect of the invention there is provided a composition comprising a vWF-cp polypeptide comprising (a) an amino acid sequence selected from the group of SEQ ID. NO 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4 and SEQ ID NO. 5, or (b) an amino acid sequence having at least 80% identity to the amino acid sequence of 30 (a).

The composition of the invention comprising the polypeptide may further comprise a divalent metal ion

selected from the group of Ca^{2+} , Sr^{2+} , Ba^{2+} and Mg^{2+} .

A further aspect of the present invention relates to a complex of isolated vWF-cp polypeptide having vWF-cp activity, vWF and a metal ion selected from the group 5 consisting of Ca^{2+} , Sr^{2+} , Ba^{2+} and Mg^{2+} . The preparation may comprise divalent metal ions in a concentration of about 1 to 10^6 ions per polypeptide molecule with vWF protease activity and can contain vWF -cp polypeptide in an essentially purified form.

10 The composition may comprise clusterin or an analog or derivative thereof having clusterin activity. With relation to the activity of the protein, the term "derivative" or "analog" of clusterin refers to a polypeptide that show the same characteristics as the native clusterin protein.

15 Clusterin is a heterodimeric glycoprotein consisting of two non-identical subunits, with a molecular mass of approximately 80 kDa (Rosenberg et al. 1995, Int. J. Biochem. Cell Biol. 27: 633-645; Tschopp et al. 1994, Clin. Exp. Immunol. 97(Suppl. 2): 11-14). It is produced in a 20 wide array of tissues and found in most biologic fluids. The physiologic functions described in the prior art include complement regulation, lipid transport, sperm maturation, initiation of apoptosis, endocrine secretion, membrane protection and promotion of cell interactions. It 25 has been found that the unusually high stability of the vWF -cp polypeptide of the present invention in circulating plasma is associated with the presence of clusterin and that the half-life of vWF cleaving protease activity *in vivo* is between 1 and 4 days, while other proteases in 30 plasma have half-lives in the range of seconds to hours. The ratio of clusterin to vWF protease polypeptide in a

composition according to the present invention is preferably in a range of 10M:1M to 1M:10M, and more preferably the ratio of clusterin and vWF is in the equimolar range. In human plasma, the concentration of vWF-cleaving protease is 2 - 10 mg/liter whereas that of clusterin is 50 - 400 mg/liter plasma (the molar ratio of vWF-cleaving protease to clusterin in human plasma is about 1:20 - 1:100).

10 In accordance with one of the objects of the invention there is provided a nucleic acid molecule comprising a nucleic acid sequence encoding vWF-cp polypeptide having (a) an amino acid sequence selected from the group of SEQ ID. NO 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4 and SEQ 15 ID NO. 5, or (b) an amino acid sequence having at least 80% identity to the amino acid sequence of (a).

The term "nucleic acid molecule" means a polynucleotide and generally refers to any DNA or RNA, which also includes variants of the DNA or RNA, the DNA or 20 RNA which may be modified or unmodified, single- and double stranded or a mixture thereof, or a short polynucleotide, generally referred to as oligonucleotides. A typical variant of a polynucleotide differs in nucleotide sequence from the original polynucleotide in sequence and may or may 25 not alter the amino acid sequence of the polypeptide encoded by the reference polynucleotide. Nucleotide changes may result in amino acid substitutions, additions, deletions, fusions or truncations in the polypeptide sequence of the polypeptide. The polynucleotide sequence 30 may be modified to improve recombinant expression. Such modification includes use of highly translated codons. Nucleic acid sequence which do hybridize with the DNA

sequence as shown in Fig. 5 or partial sequence encoding an amino acid of at least 12 amino acid are within the scope of the present invention. Hybridization Techniques are well known to the skilled artisan.

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According to another aspect of the invention there is provided an expression vector comprising a nucleic acid molecule comprising a nucleic acid sequence encoding a vWF-cp polypeptide having (a) an amino acid sequence of selected from the group of SEQ ID. NO 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4 and SEQ ID NO. 5, or (b) an amino acid sequence having at least 80% identity to the amino acid sequence of (a).

The nucleic acid molecule of the invention can be used for constructing expression systems providing appropriate elements for replication of vector within a host cells and expression of the DNA which can then be used for the expression of a polypeptide having vWF cleaving protease activity according to the present invention. The nucleic acid sequence may be modified to improve expression, such as addition of a Kozak sequence.

The expression vector may comprise, for example, in the direction of transcription, a transcriptional regulatory region and a translational initiation region functional in a host cell, a DNA sequence comprising a polynucleotide expressing a VWF -cp polypeptide according to the present invention and translational and transcriptional termination regions functional in said host cell, wherein expression of the nucleic sequence is regulated by the initiation and termination regions. The expression vector may also contain elements for the replication of the nucleotide. Examples of DNA expression

vectors are pBPV, pSVL, pRc/CMV, pRc/RSV, myogenic vector systems, pcDNA3 based vector (Invitrogen) or vectors derived from viral systems, for example from vaccinia virus, adenoviruses, adeno-associated virus, herpes viruses, retroviruses or baculo viruses. Suitable mammalian expression vectors usually contain one or more eukaryotic transcription units that are capable of expression in mammalian cells. The transcription unit is comprised of at least a promoter element to mediate transcription of foreign DNA sequences. Suitable promoters for mammalian cells are known in the art and include viral promoters such as that from simian virus 40 (SV40), cytomegalovirus (CMV), Rous sarcoma virus (RSV), adenovirus (ADV), bovine papilloma virus (BPV) or other promoters selected from the group of methallothionein promoter and β -Actin promoter. Other promoters known in the art are also applicable for expression.

The expression vector containing the nucleic acid sequence which encodes the vWF-cp polypeptide according to the present invention can be used to transform host cells which then produce the polypeptide. The transformed host cells can be grown in a cell culture system to produce the polypeptide *in vitro*. The host cells can excrete the polypeptide having vWF protease activity into the cell culture medium from which it can be prepared or the polypeptide can be isolated from the cell lysate.

The expression vector comprising the nucleic acid molecule encoding a polypeptide comprising (a) an amino acid sequence of selected from the group of SEQ ID. NO 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4 and SEQ ID NO. 5, or (b) an amino acid sequence having at least 80% identity

to the amino acid sequence of (a) can be transformed or transfected into a host cells for expression of the recombinant polypeptide. Suitable host cells include any cell capable of producing vWF -cp polypeptide after being 5 transformed or transfected. Preferred cells include bacterial cells, yeast, insect cells or animal cells. The host cell may be a cell derived from the body of a mammal, for example fibroblasts, keratinocytes, hematopoietic cells, hepatocytes or myoblasts, which are transformed *in* 10 *vitro* with an expression vector system carrying a nucleic acid according to the present invention and re-implanted into the mammal. The polypeptide according to the present invention encoded by said nucleic acid will be synthesized 15 by these cells *in vivo* and they will exhibit a desired biological activity in the mammal.

Mammalian cell lines available as hosts for expression are known in the art and include many immortalized cell lines available from the American Type Culture Collection (ATCC). Exemplary mammalian host cells include particularly 20 primate cell lines and rodent cell lines, including transformed cell lines. Preferably for stable integration of the vector DNA, and for subsequent amplification of the integrated vector DNA, both by conventional methods, Chinese hamster ovary (CHO) cells are employed as a 25 mammalian host cell of choice. Other suitable cell lines include, but are not limited to, HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS-1), human hepatocellular carcinoma cells (e.g., Hep G2), human adenovirus transformed 293 cells, HEK 293cells, SKHep 30 cells, mouse L-929 cells, HaK hamster cell lines, murine 3T3 cells derived from Swiss, Balb-c or NIH mice and a number of other cell lines. Another suitable mammalian cell

line is the CV-1 cell line. Normal diploid cells, cell strains derived from in vitro culture of primary tissue, as well as primary explants, are also suitable. Candidate cells may be genotypically deficient in the selection gene, 5 or may contain a dominantly acting selection gene.

Host cells can be either transformed cells or untransformed cells. The host cells are preferably those expressing furin either naturally or after being genetically engineered to express recombinant furin.

10 The selection of suitable mammalian host cells and methods for transformation, culture, amplification, screening and product production and purification are known in the art.

15 The host cell transformed with a vector carrying the vWF-cp polypeptide en coding nucleic acid may then be cultured under suitable conditions if desired, with amplification introduced genes. Effective conditions include, but are not limited to, appropriate media, 20 bioreactor, temperature, pH and oxygen conditions that permit protein production. The method of this present invention therefore comprises culturing a suitable cell or cell line, which has been transformed with a nucleic acid sequence encoding a amino acid sequence of the vWF-cp 25 polypeptide, the coding sequence under the control of a transcriptional regulatory sequence. The expressed polypeptide is then recovered, isolated and purified from the culture medium if secreted by the cells or from the cell, if expressed intracellularly by appropriate means 30 known to one of skill in the art.

The nucleic acid molecule of the invention or a fragment thereof can be expressed in a eukaryotic or

prokaryotic microorganism system, such as fungi, including yeast, or bacteria. Fragments can include truncated forms of the vWF cleaving protease. Examples of truncation include, but are not limited to, N-or C-terminal deletions.

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The nucleic acid molecule of invention may also be used to generate transgenic animals, which express said polypeptide *in vivo*. In one embodiment of this specific application, the transgenic animals may express the vWF -cp polypeptide in endogenous glands, for example in mammary glands from which the said proteins are secreted. In the case of the mammary glands, said vWF protease polypeptide is secreted into the milk of the animals from which said proteins can be prepared. The animals may be mice, cattle, pigs, goats, sheep, rabbits or any other economically useful animal.

The vWF-cp polypeptide of the invention can be used in a method of purification of vWF by providing as a ligand for vWF a vWF-cp polypeptide of the invention, contacting a solution comprising vWF with the vWF-cp polypeptide ligand under conditions whereby vWF is bound to the ligand and recover from said ligand purified vWF. The vWF-cp polypeptide ligand may be bound to a solid carrier.

25

Furthermore, the vWF-cp polypeptide of the invention can also be used for the development of vWF-cp polypeptide binding molecules using techniques known in the art. The binding molecules can be anti-vWF-cp polypeptide antibodies which can be produced by immunization of an animal with a polypeptide of the invention and isolation of anti-vWF-cp polypeptide antibodies from the animal.

"Antibodies" as used herein includes polyclonal and monoclonal antibodies, chimeric antibodies, single chain, and humanized antibodies, as well as Fab fragments, including the products of an Fab or other immunoglobulin 5 expression library, peptides or peptidomimetics. Monoclonal antibodies can be produced according to methods well known in the art. Other binding molecules can be derivatives of a antibody, such as a single chain antibody, a Fab-, or Fab'2-fragment, a chimeric antibody, or a peptide or a 10 peptidomimetic, which can be obtained by known methods such as e.g. the phage display method.

Within the aspect of the invention, a vWF-cp polypeptide binding molecule selected from the group of single chain antibodies, Fab- or Fab'2-fragment or 15 polypeptides with vWF protease binding site can be produced by a method, wherein a phage display library is screened for anti-vWF-cp polypeptide binding molecule, the nucleic acid sequence of positive clones is determined and a nucleic acid molecule comprising the sequence is cloned 20 into an expression vector.

The development of the antibodies, antibody derivatives, peptidomimetics or any other molecule which binds vWF-cp polypeptide can be accomplished according methods known to the prior art (Greer et al. 1994, J. Med. Chem. 37: 1035- 25 1054; Kemp 1990, Trends Biotechnol. 8: 249-255).

The vWF-cp polypeptide binding molecule of the invention may be used as a ligand to purify vWF-cp. Such a method can be performed by contacting a solution comprising 30 vWF-cp polypeptide with the vWF-cp polypeptide binding molecule under conditions whereby vWF-cp polypeptide is bound to the binding molecule and recovering purified vWF-

cp polypeptide by selectively eluting vWF-cp polypeptide from the vWF-cp binding molecule. The vWF-cp binding molecule may be bound to a solid carrier.

5 The vWF-cp polypeptide binding molecule can be also be used in a method for detection of vWF-cp polypeptide in a sample. Thereby a solution suspected to contain vWF-cp polypeptide is contacted with a vWF-cp polypeptide binding molecule as described above under conditions allowing the 10 formation of a complex of vWF-cp polypeptide/vWF-cp polypeptide binding molecule and detection of the complex.

The vWF-cp polypeptide of the invention can be used, for example, to process plasmatic or recombinantly produced 15 vWF. Recombinant vWF (r-vWF) can be produced in CHO cells, e.g. according to Fischer et al. (1995. FEBS Lett 375: 259-262). The r-vWF recovered in this manner is available as a mature vWF and has a singlet structure, i.e. it differs from plasma-derived vWF, which always has a characteristic 20 satellite structure when examined on 2% SDS agarose gels. US5,854,403 teaches that r-vWF is comprised of multimers with high structural integrity which is retained even after purification and treatment for the inactivation of viruses. The intact structure of the r-vWF is defined by a result of 25 electrophoretic analysis consisting of multimer bands with an absence of satellite bands. To prepare an r-vWF preparation having a structure more closely corresponding to that of plasma-derived vWF from r-vWF with singlet structure, r-vWF is treated with the vWF protease polypeptide or a composition comprising the vWF-protease 30 polypeptide of the present invention, and optionally metal ions and/or clusterin.

According to one aspect of the invention, the vWF-cp polypeptide can be used for the production of a preparation for the prophylaxis and therapy of diseases that show supranormal vWF content or an increased level of high-
5 molecular weight vWF in patients, such a thromboembolic disease. This can result in thromboses and thromboembolic diseases. For example, thrombotic thrombocytopenic purpura (TTP), Henoch-Schönlein purpura, preeclampsia, neonatal thrombocytopenia or haemolytic-uremic syndrome. By
10 administering an effective dose of a polypeptide of the invention and having a vWF protease activity, this can lead to reduction of the content of high molecular weight vWF multimers in the patients, resulting in effective therapy of these diseases. The disease can be selected from the
15 thromboembolic disease is thrombotic thrombocytopenic purpura (TTP), Henoch-Schönlein purpura, preeclampsia, neonatal thrombocytopenia or hemolyticuremic syndrome.

The invention is described in the following examples,
20 without being limited thereto.

25

30

Examples**Example 1:****Isolation of vWF-cp polypeptides**

5 1.1. Preparation of an IgG-eTTP-coupled affinity gel. The IgG-eTTP is isolated by aid of a 20 ml protein A-Sepharose® (diameter 1.6 cm) in TBS, pH 7.4. Pheresis plasma of a patient suffering from acquired TTP ("erworbenes" TTP; eTTP), which previously had been assayed
10 for its inhibitor content relative to the vWF-cleaving protease is applied to the column in a volume of 50 ml. After subsequent rinsing with TBS, pH 7.4, the bound IgGs are step-wise eluted with citrate, 0.1 M, pH 4.0, and glycine, 0.1 M, pH 2.7. The fractions immediately are
15 brought to a physiological pH by adding Tris, 1.5 M, pH 8.8, and dialysed against TBS, pH 7.4. The Affi-Gel® Hz is coupled according to the producer's instructions with the IgG-eTTP which had been ished out of the protein A-Sepharose® with a pH of 4.0. The column material prepared
20 in this manner first is ished as prescribed, subsequently it is washed 3 times alternatingly with 50 ml of buffer B and 200 ml of buffer A (chapter 1.7). Prior to use, intensive rinsing with buffer A is carried out in each instance.

25

1.2 First Step

As the starting material, 100 ml of pooled CPD plasma which had come from at least three donors and had been stored at -20°C, is used after centrifuging at 2,500 rpm
30 (1,100g) for 5 min. At a relatively low flow rate (FR: 30 ml/h), the plasma is loaded on a 200 ml chromatographic

column with IgG-eTTP Affi-Gel® Hz (hydrazide, diameter 2.6 cm) which had been equilibrated in buffer A. After washing with at least 400 ml of buffer A over night at the same flow rate, a 200 ml desalting gel filtration column (Bio-5 Gel® P-6DG, diameter 2.6 cm) and a 10 ml protein G-Sepharose® (diameter 1.6 cm), which previously also had been rinsed with buffer A, is connected thereto. After the flow rate had been increased to 100 ml/h, the proteins bound to Affi-Gel Hz are eluted directly with 50 ml of buffer B onto the Bio-Gel® P-6DG so as to remove from the proteins the NaSCN that had been in buffer B. The proteins which had been eluted from the desalting column prior to the NaSCN are led through the protein G Sepharose® without interruption, where they are freed from the IgGs. Here, the flow rate is lowered to 50 ml/h so as to extend the dwell time of the proteins in the 10 ml column. For regeneration, the protein G-Sepharose® is shortly washed with buffer C, and the eluted IgG fraction is stored for analysis.

The first step is carried out 8 times before the 20 collected fractions which had been frozen at -20°C are pooled and further processed.

1.3 Second Step

The pooled fractions from 8 chromatographies of the 25 first step are diluted 1:1 with H₂O so as to obtain an ionic strength at which the desired proteins would bind to the anion exchange column (High Q Support®). The sample whose volume is from 1,500 to 1,800 ml, depending on the charge used, is checked for its pH and its ionic strength 30 and applied over night at a FR of 90 ml/h through a 50 ml

column with Therasorb® (diameter 1.6 cm) onto a 5 ml High Q Support® (diameter 1.6 cm). Both, Therasorb and High Q Support® had previously been equilibrated in buffer D. After washing with approximately 150 ml of buffer D, the 5 Therasorb is disconnected, and the 25 ml Lentil Lectin Sepharose® (diameter 1.6 cm) which had been equilibrated in buffer E is connected to follow the High Q Support®. At a FR of 60 ml/h, the proteins bound to High Q Support are immediately eluted with buffer E directly to the Lentil 10 Lectin-Sepharose®. The proteins which bound to the Lentil Lectin-Sepharose® can be eluted in two steps with buffers G and H and can be collected. The proteins which had remained bound to Therasorb and High Q Support® are washed 15 out with buffer C or buffer F, respectively, and discarded after an analysis.

For regeneration, before being used, the Lentil Lectin-Sepharose® in each case is rinsed according to the producer's instruction 3 times alternatingly with 20 ml each of buffers I and J, the High Q Support is rinsed 20 successively with 10 ml each of NaOH 1N and NaCl 1M.

1.4 Third Step

The pooled fractions which had been eluted from the Lentil Lectin-Sepharose® with buffer H are dialysed three 25 times for a total of 4 h, each against 1 l of buffer D, and again applied to the High Q Support at a flow rate of 60 ml/h. Connected therein front is a 5 ml heparin-Sepharose (diameter 1.4 cm), which likewise had been equilibrated in buffer D. After the application of the sample, it is rinsed 30 with approximately 50 ml of buffer D, the heparin-Sepharose

is disconnected, and a 500 ml Sephacryl® S-300 HR (diameter 2.6 cm), which had been equilibrated in buffer L, is connected thereto. The proteins bound to High Q Support are directly eluted to the gel filtration column with 10 ml 5 of buffer K. The exclusion chromatography is effected at a flow rate of 42 ml/h, and the fractions are collected at 7 ml each. The proteins which are more strongly bound to High Q Support® are again eluted with buffer F, those which remained adhered to the heparin-Sepharose, with 10 buffer K.

1.5 Fourth Step

The pool of the active fractions from the third step is applied without treatment at a FR of 10 ml/h to a 1 ml 15 anti- α_2 -macroglobulin column (flow rate 0.7 cm) which had been equilibrated in buffer L. The anti- α_2 -macroglobulin column is prepared by immobilization according to the instructions, of rabbit-anti- α_2 -macroglobulin antibodies at a concentration of 4.9 mg/ml on CNBr-activated 20 Sepharose. The proteins bound thereon are eluted with NaSCN 3M in buffer L and with buffer C and stored for analysis.

Table 1: List of buffers

buffer A	Tris	10 mM	pH 7.4
	NaCl	0.15 M	
	Na ₃ -citrate	1mM	
	Na acid	0.02%	
buffer B	NaSCN in buffer A	3.0M	pH 7.4
buffer C	glycine	0.1M	pH 2.7
	Na acid	0.02%	
buffer D	Tris	10mM	pH 7.4
	NaCl	75mM	
buffer E	Tris	20mM	pH 7.4
	NaCl	0.5M	
	MnCl ₂	1 mM	
buffer F	Tris	10mM	pH 7.4
	NaCl	1.0M	
buffer G	Tris	20mM	pH 7.4
	NaCl	0.5M	
	Methyl- α -D- mannopyranoside	30mM	
buffer H	Tris	20mM	pH 7.4
	NaCl	0.5M	
	Methyl- α -D- mannopyranoside	0.3M	
buffer I	Tris	20mM	pH 8.5
	NaCl	0.5M	
buffer J	Na acetate	20mM	pH 5.5
	NaCl	0.5M	
buffer K	Tris	10mM	pH 7.4
	NaCl	0.5M	
buffer L (TBS)	Tris	10mM	pH 7.4
	NaCl	0.15M	

Table 2: List of Chromatographic materials

Material	Provider /Company
affi-gel hydrazide gel®: for immobilizing specific IgG's	Bio-Rad, Hercules, CA, USA
anti- α_2 -macroglobulin column: isolation of α_2 -macroglobulin	applicant's own production (see 1.5): rabbit-anti-human- α_2 -macroglobulin antibody on CNBr activated Sepharose 4B; 4.9 mg/ml
Bio-Gel® P6-DG, medium: gel filtration with exclusion limit > 6kDa	Bio-Rad
CNBr activated Sepharose 4B®: for immobilizing proteins	Amersham Pharmacia Biotech, Uppsala, S
heparin Sepharose, HITrap® 5ml: affinity chromatography: binds various proteins	Amersham Pharmacia
High Q Support®, Macro-Prep: strong anion exchanger	Bio-Rad
IgG-eTTP Affi-gel Hz: for binding vWF-protease	applicant's own production (see 1.1.): IgG-eTTP on Affi-gel Hz hydrazide
Lentil Lectin-Sepharose 4B: affinity chromatography: binds to sugar residues of proteins	Amersham Pharmacia
protein A Sepharose® CL-4B. binds IgG of type 1, 2 and 4	Amersham Pharmacia
protein G Sepharose® 4FF: isolation of IgGs of all types	Amersham Pharmacia
Sephacryl® S-300 HR: gel filtration for MWs 10,000 to 1,500,000	Amersham Pharmacia
Therasorb: coupled with sheep-anti-human-Ig-antibodies: isolation of human immunoglobulins	Serag-Wiessner, Naila, D

1.6 *Fifth Step*

Alternatively, or in addition to step four, an anti-clusterin column chromatography as a further step can be applied. The samples are prepared identically to the anti-
5 α 2- macroglobulin-column using anti-clusterin antibodies.

1.7. SDS-Page reduced/non-reduced

The final preparation from the third step of isolation is electrophoresed on a 1.5 mm-thick SDS-polyacrylamide gel
10 according to Laemmli (1970, *Nature*, 227: 680-685). A gradient of 4 to 12% polyacrylamide is used for fractionation of proteins. After electrophoresis under non-reducing or reducing conditions (final concentration 65 mmol/l dithiotreitol DTT), the proteins are made visible
15 by silver stain or Commassie blue stain (Fig. 2). Under non-reducing conditions bands having a molecular weight (MW) of about 150 kD, 140 KD, 130 KD and 110 KD are detectable and under reducing conditions of about 180 KD, 170 KD, 160 kD and 120 KD.

20

1.8 Determination of vWF protease activity

The different fractions isolated by the purification process as described above are tested for vWF cleaving activity by the method as described by Furlan et al.
25 (1996, *Blood* 87: 4223-4234).

30

Example 2:**Amino acid sequencing and amino acid analysis of the vWF-cp polypeptide**

5 The final protein preparation from the third step of isolation is electrophoresed on a 1.5 mm-thick SDS-polyacrylamide gel according to Laemmli (1970, *Nature*, 227: 680-685). A gradient of 4 to 12% polyacrylamide is used for fractionation of high molecular weight proteins, and a
10 gradient of 8 to 12% polyacrylamide for low molecular weight proteins. After electrophoresis under non-reducing or reducing conditions (final concentration 65 mmol/l dithiotreitol), the proteins are blotted onto PVDF-membranes and stained for 2 min with 0.25% Coomassie Blue
15 in 45% methanol, 9% acetic acid and 46% H₂O. After rinsing with a mixture of 50% methanol, 10% acetic acid and 40% H₂O, the visible protein bands are cut out and analyzed on a Procise-CLC Sequencer (Foster City, CA) at the Chemical Institute of the University of Bern.
20 The N-terminal amino acid sequence of polypeptide bands separated by SDS-PAGE of purified vWF-cleaving protease is shown in Table 3.

25

30

Table 3: Determination of N-terminal amino acid sequence of vWF-cp

Molecular weight	Amino Acid sequence
350 kDa unreduced	Ser-Val-Ser-Gly-Lys-Pro-Gln-Tyr-Met-Val*
150 kDa unred.	Ala-Ala-Gly-Gly-Ile
140 kDa unred.	Ala-Ala-Gly-Gly-Ile
130 kDa unred.	Ala-Ala-Gly-Gly-Ile
110 kDa unred.	Ala-Ala-Gly-Gly-Ile-Leu-His-Leu-Glu
70 kDa unred.	Asp/Ser-Gln/Leu-Thr/Met-Val/Pro-Ser/Phe**
180 kDa reduced	Ala-Ala-Gly-Gly-Ile-Leu-His-Leu-Glu
170 kDa reduced	Ala-Ala-Gly-Gly-Ile
160 kDa reduced	Ala-Ala-Gly-Gly-Ile-Leu-His-Leu-Glu-Leu-Leu-Val-Ala-Val-Gly
120 kDa reduced	Ala-Ala-Gly-Gly-Ile-Leu-His-Leu-Glu-Leu-Leu-Val-Ala-Val-Gly
40 kDa reduced	Asp-Gln-Thr-Val-Ser**

* identified as α_2 -macroglobulin

5 ** identified as clusterin

Analysis of the composition of the amino acids is performed from the same sample as used for amino acid sequencing. The protein bands are hydrolyzed in the gas phase over 6 N HCl for 22 hours at 110 °C and the amino acids are determined by high-performance liquid chromatography. Four unreduced polypeptide bands from SDS-PAGE of purified vWF-cp with M_r 150, 140, 130, and 110 kDa are analyzed. The results are shown in Tab. 4.

Table 4: Amino acid composition of isolated vWF-cp polypeptide

Amino acid	No. residues/100 residues			
	150 kDa	140 kDa	130 kDa	110 kDa
Asx	6.7	7.0	7.4	8.3
Glx	12.2	12.0	12.8	11.8
Ser	8.4	8.9	8.8	9.2
Gly	11.8	12.1	12.1	12.9
His	2.5	2.3	2.5	2.4
Arg	8.3	7.6	8.1	7.2
Thr	5.6	5.5	5.6	5.7
Ala	10.1	9.5	9.6	8.2
Pro	8.9	8.5	8.3	8.1
Tyr	2.1	2.7	2.3	2.4
Val	7.0	6.9	6.7	6.5
Ile	2.7	2.9	2.6	3.0
Leu	10.0	9.9	9.1	9.7
Phe	2.6	2.8	2.6	3.2
Lys	1.0	1.3	1.3	1.4

Example 3:**Identification of the vWF-cp gene**

The nucleic acid coding sequence of the amino acid sequence of peptide AAGGILHLELLVAVG (SEQ. ID. NO. 2) of the N-

5 terminal 15 residues of the purified plasmatic human vWF-cp is determined and corresponds to the nucleic acid sequence GCT GCA GGC GGC ATC CTA CAC CTC GAG CTG CTG GTG GCC GTG GGC (SEQ. ID. No. 9). A database searching in the NCBI GenBank (<http://www.ncbi.nlm.nih.gov/>) showed the location of the 10 corresponding nucleic acid sequence GCT GCA GGC GGC ATC CTA CAC CTC GAG CTG CTG GTG GCC GTG GGC (SEQ.ID.No. 9) on chromosome 9 clone RP11-244N20 (AL158826, GI11544459). Thus the nucleotide sequence from base 150001 to 185911 is screened for potential exons. Consecutive overlapping 15 genome-segments with various lengths (1500 bases-5000 bases) are analysed using search engines that are queried via the internet-explorer. The genomic sequence segments, its translations and the results of the search are managed using the 'Vectors NTI Suitel v.5.2' computer-program 20 (Informax Inc., USA). The sequence of RP11-244N20 has a size of approximately 185 kb and the identified nucleic acid sequence starts at position 156.653. Use of an exon search program (Grail, <http://compbio.ornl.gov/Grail-1.3>) allowed identification of about 30 putative exons upstream 25 and downstream of position 156.653. The first four exons of the search are translated into the corresponding amino acid sequence and these sequences are searched for homologies. This search revealed that each sequence displays high homology with the amino acid sequence of the family of 30 human disintegrin and metalloproteinases with thrombospondin motifs (ADAM-TS).
In order to connect the putative exons poly-A -RNA from

liver is reverse transcribed and the cDNA is then PCR amplified with primers specific for some of the putative exons (Table 5). The PCR products of primers 6142 (SEQ.ID.No.16)-6277 (SEQ.ID.No.17), 6142 (SEQ.ID.No.16)-5 6546 (SEQ.ID.No.18), 6351 (SEQ.ID.No. 19)-6546 (SEQ.ID.No. 18), 6278 (SEQ.ID.No. 21)-6407 (SEQ.ID.No. 20), 6346 (SEQ.ID.No. 23)-6395 (SEQ.ID.No. 24) and 6406 (SEQ.ID.No. 25)-6506 (SEQ.ID.No. 26) (derived from putative exons 1-6, 3-11, 6-11, 10-14, 13-16, 14-23, respectively) revealed 10 bands of the expected size. After sequencing an open reading frame from exon 1 to exon 23 located near to the 3' end of clone RP11-244N20 can be identified. The 3' end of the cDNA sequence is examined by PCR amplification of the cDNA using forward primer 6548 (SEQ.ID.No. 27) of exon 22 15 and the specific MC18 sequence of the RT primer spdT. The resulting fragment of a size approximately to 1.8 kb is sequenced and after database search the 3' part of the new cDNA sequence can be located on region q34 (AC002325) on chromosome 9.

20 The unknown sequence of the gene at the 5' end is identified by 5' -RACE and sequencing of the resulting PCR product revealed two additional exons. None of the next five exons upstream of exon 1-2 can be connected by PCR to the already known cDNA sequence. All together 29 exons with 25 a cDNA size of 4.6 kb are found. The vWF -cleaving protease gene spans approximately 37 kb, a schematic drawing of the localization of the exons is shown in Figure 4. The complete cDNA sequence is depicted in Figure 5.

Table 5 Sequences of Primers Used for Reverse Transcription, PCR and Cloning

				production of cDNA
		spdT	5' -GAGCAAATTCCCTGTACTGAC (T) 30 NN-3' (SEQ.ID.No. 12)	
		MC18	5' -GAGCAAATTCCCTGTACTGAC-3' (SEQ.ID.No. 13)	specific part of spdT
5	6434		5' -GACCACGGGTATCGACGTGAC-3' (SEQ.ID.No. 14)	anchor Primer
	6275		5' -CTCAGGGTTGATGGTCTGGCT-3' (SEQ.ID.No. 15)	
	6142		5' -CGGGCTGCAGGGGGATCCATACACCTGGAG-3' (SEQ.ID.No. 16)	exon 5 reversed
	6277		5' -AATGGTGTACTCCAGGTCGAG-3' (SEQ.ID.No. 17)	exon 3 forward
	6546		5' -TGGAGGTAGCCACCAACACA-3' (SEQ.ID.No. 18)	exon 6 reversed
10	6351		5' -GAGTTGCCTGTATGGTAACCG-3' (SEQ.ID.No. 19)	exon 11 reversed
	6407		5' -GAGCCCCCTCCGGGGCTGCA-3' (SEQ.ID.No. 20)	exon 6 forward
	6278		5' -CGCTCCCTGGTGGAGCTGACCC-3' (SEQ.ID.No. 21)	
	6561		5' -CTCACCATCGTCCTCCCCAT-3' (SEQ.ID.No. 22)	exon 14 reversed
	6346		5' -ATCATGAAGCGTGGAGACAGC-3' (SEQ.ID.No. 23)	exon 10 forward
	6395		5' -CCTGGGGGGTCCCCAGATG-3' (SEQ.ID.No. 24)	exon 23 reversed
15	6406		5' -TGCAGGCCACGGAAAGGGCTC-3' (SEQ.ID.No. 25)	exon 13 forward
	6506		5' -CAGGGCTCCAGGCTGCAAGC-3' (SEQ.ID.No. 26)	exon 16 reversed
	6548		5' -AGGAAGAGCTGTGGCCTG-3' (SEQ.ID.No. 27)	exon 14 forward
	6725		5' -GACGGGGCCAGCCGGGGCTGCAGGGGGCATCTACAC-3' (SEQ.ID.No. 28)	exon 23 reversed
20	6276		5' -GGCCCTCGAGGGTTCCCTTCCAGG-3' (SEQ.ID.No. 29)	exon 22 forward
				exon 29 reversed -XbaI

Determination of Similarities to Other Proteins

The SMART program (<http://smart.embl-heidelberg.de>) is searched for domain architectures of the protease and identified protein modules including a signal peptide with its

5 transmembrane region, a furin cleavage site, a metalloprotease domain, i.e. a Zn -protease catalytic site consensus sequence (HEXXH), a Met turn, a disintegrin domain, a cysteine -rich region, and thrombospondin type 1 (TSP-1) motifs (Figure 6).

These modules are also shared by members of the novel ADAMTS

10 (for a disintegrin and metalloprotease with thrombospondin type 1 motif) metalloproteinase family (Tang 1999. FEBS Lett. 445:223-225; Tang 2001. Int. J. Biochem. Cell Biol. 33:33-44).

The amino acid sequence of the vWF cleaving protease is

aligned with all the human ADAMTS protein sequences, which are

15 listed by the human genome organization (HUGO) gene nomenclature committee

<http://www.gene.ucl.ac.uk/nomenclature/genefamily/adamts.html>

A schematic representation of the domains is shown in Figure

6. In contrast to the ADAMTS proteins our putative new member

20 of this family lacks a prodomain. However, examination of the amino acid sequences of the next five putative exons upstream of exon 1-2 showed no sequence homologies to ADAMTS proteins.

In addition, no signal peptide sequences can be found on these exons. We thus named the vWF -cleaving protease ADAMTS13.

25 The deduced amino acid sequence is also compared with the EMBL protein database (<http://ww2.ebi.ac.uk>). Exons 9 to 16

correspond partly to an already submitted hypothetical 39.9

kDa protein located on chromosome 9 and termed open reading

frame 8 (C9ORF8, XM 005647, GI12735207). In addition exon 26

30 to 29 correspond to the hypothetical protein DKFZp434C2322

(NM032252, GI14149974) (Wiemann et al. 2001. Genome Res. 11:

422-435).

Example 4:**Affinity purification of von Willebrand Factor (vWF)**

The peptide with the sequence AAGGILHLELLV (SEQ.ID.No.

1) is synthesized on a solid-phase support following the
5 method of Barany et al. (1980. Solid-phase Peptide
Synthesis. In: *The Peptides*. vol. 2 ,Gross, E. and
Meienhofer, J. (eds.) Academic, New York, SEITEN). After
cleavage and de-protection of the peptide, the peptide is
10 purified by ion-exchange chromatography. The peptide is
characterized by reverse phase HPLC on a C8 silica column
with gradient elution in trifluoro acetic acid with
acetonitrile. The peptide showed no major byproducts.

The peptide is solubilized in a concentration of
5 mg/mL in 0.1 molar phosphate buffer pH 7.5 and incubated
15 with a pre-activated gel suitable for affinity
chromatography (Actigel, ALD-Superflow, Sterogene). Prior
to coupling of the peptide to the gel the pre-activated
matrix is excessively washed with the same phosphate
buffer. One volume of the pre-washed gel is then mixed with
20 one volume of the peptide solution to be immobilized and
subsequently 0.1 volume portions of a solution of 0.1 molar
cyanoborohydride (NaCNBH₃) in 0.1 molar phosphate buffer pH
7.5. The gel is suspended in this solution and shaked for
15 hours at room temperature. Subsequently the gel is
25 washed on a sinter funnel with a 10-fold volume of the
phosphate buffer containing 150 mmolar NaCl and with
5 volumes of the phosphate buffer containing 2 molar NaCl.
Then the gel is equilibrated with an access of 0.1 molar
phosphate buffer pH 7.0.

30 The gel is then transferred into a chromatographic
column having a dimension of diameter to gel bed height of

1:4. By determining the peptide concentration the solution of the incubation supernatant after separation from the gel and the washing solutions the amount of peptide coupled to the affinity matrix is calculated. The coupling rate is
5 85%.

The gel is subsequently used to purify vWF from a Factor VIII (FVIII)/vWF complex. A FVIII/vWF complex concentrate is produced according to US 4,814,435 containing vWF in a concentration of 260 U vWF:Ag/ml and a
10 specific activity of 13.5 U vWF:Ag/mg Protein. The concentrate is diluted with 20 mM phosphate buffer pH 7.0 to a final vWF concentration of 6 U vWF:Ag/mL. A volume of 20 ml of this solution is subjected to the affinity column with immobilized peptide described above. After washing the
15 column with 10 ml of the phosphate buffer the vWF specifically bound to the peptide ligand is eluted by a linear gradient from 0-2 mol/l NaCl in phosphate buffer at a flow rate of 1 ml / minute. Fractions of 1 ml are collected and their optical density is determined at
20 280 nm. All fractions are measured for their content of vWF antigen determined by a specific ELISA method (Asserachrom vWF, Boehringer, Mannheim). Measurement showed a specific peak of vWF eluting from the peptide at a NaCl concentration of 100 mmol/l, while most of the protein
25 measured by UV-absorption eluted prior to the vWF fraction with the washing buffer. The vWF containing fractions are pooled and measured for vWF activity. The vWF in this pool had a specific activity of 95 U vWF/mg protein and is essentially free from other proteins.

30

Example 5:**Anti-vWF-cp polypeptide antibodies**

The peptide with the sequence AAGGILHLELLV (SEQ.ID.No.

1) is synthesized and purified as described in example 4.

5 The peptide is then used to immunize 3 months old BALB/c mice with the following protocol: A primary subcutaneous injection of 100 µg peptide antigen emulsified in Freund's complete adjuvant in 100 µl followed by intra-peritoneal boosts of 100 µg peptide antigen in phosphate buffered saline at monthly intervals.

10 The anti-peptide titer is tested by routine ELISA method using purified peptide as screening antigen. After the final boost the spleens are taken from the mice for cell fusion. Cell fusion is carried out according to a 15 standard protocol originally described by Köhler et al. (1975, Nature 256:495-497). Anti-peptide antibodies producing hybridoma cell lines are screened by standard techniques with the purified peptide as screening antigen essentially based on a conventional ELISA methodology.

20 After cloning a cell line can be isolated with a high expression level of an antibody specific for the screening peptide with the sequence AAGGILHLELLV. This cell line is cultured on serum-free culture medium and grown to high density. The supernatant of the cell culture is harvested 25 by centrifugation to remove cells and the monoclonal antibody containing supernatant is concentrated by ultra-diafiltration and conditioned for further use.

30 The monoclonal antibody obtained had a high selectivity for the vWF cleaving protease as described by Furlan et al. (1996, Blood 87: 4223-4234). This monoclonal antibody is immobilized to a polystyrene ELISA plate in a carbonate/bi-carbonate buffer, 0.05 molar, pH 9.6, at a

concentration of 5 µg immunoglobuline/ml overnight (16 hours) at 4°C, with each 100 µl of coating solution per well. The coating solution is removed from the wells and replaced by a solution of bovine serum albumin (BSA) at a 5 concentration of 100 µg/ml at a volume of 100 µL per well, for 2 hours. The BSA solution is removed and the wells are washed with phosphate buffered saline. The pre-coated plates are then incubated with either samples of platelet poor plasma from healthy human plasma donors or platelet 10 poor plasma from patients with an unclear diagnosis of either thrombotic thrombocytopenic purpura (TTP) or hemolytic uremic syndrome (HUS). After incubation of the plasma samples with the antibody coated ELISA plates as in a routine sandwich ELISA system, after 3 hours the plasma 15 is removed from the wells. Wells are washed with phosphate buffered saline and incubated with the monoclonal antibody directed against the peptide with the sequence AAGGILHLELLV, conjugated with horse radish peroxidase following the method of Wilson et al. (1978. In: 20 *Immunofluorescence and Related Staining Techniques*; Knapp, W. Holubar, K. and Wick, G (eds.), Elsevier/North Holland, Amsterdam, 215) and detected by the OPT reagent as described by Cathy et al, (1989. *ELISA and related enzyme immunoassays*, In: *Antibodies II a practical approach*. Cathy 25 D (ed.), IRL Press Eynsham Oxford England, 97).

Based on the level of the samples from the healthy human plasma donors a normal range is established. Plasmas from patients with HUS had a vWF protease activity equivalent to healthy humans while patients with TTP had a 30 decreased protease activity as confirmed by an assay based on a different assay principle as described in WO 00/50904.

Example 6:**Cloning of the vWF cleaving protease gene**

Human salivary gland poly A+ RNA is purchased from Clontech. First strand cDNA is obtained using Expand

5 reverse transcriptase (Hoffmann La Roche) and oligo d(T) primer according to the manufacturer's instructions. PCR is performed using 5' CGGCAGGATCCTACACCTGG 3' (SEQ.ID.NO. 10) and 5'AATGGTGACTCCCAGGTCGA 3' (SEQ.ID.NO. 11) as primers with 10 ng of salivary gland cDNA as template and 10 U of 10 Hot Star Taq polymerase (Qiagen). The thermal cycling parameters are an initial incubation at 94° C for 15 minutes followed by 45 cycles of 94° C (50 sec), 50° C (50 sec), 72° C (2 min). PCR products are directly sequenced in both directions using the BigDye Terminator Cycle 15 Sequencing Ready Reaction Kit (PerkinElmer Life Science).

The obtained DNA sequence is used to scan the genomic data base using BLAST (basic local alignment search tool) programs and matched to the chromosome 9 clone RP11-224N20. DNA sequence is translated to amino acids sequence using 20 ExPASY proteomic tools. The DNA and translated amino acid sequence corresponding to 4 putative exons of chromosome 9q34 is shown in Fig. 5.

RT-PCR fragments encompassing either the pre-prosequence or the mature domains of the vWF cleaving 25 protease are cloned into vector pDrive (Qiagen) by PCR cloning. For amplification of mature vWF-cp the primer #6601 (5'-AGCGGTCTCTATGGCTGCAGGCGGCATCCTACACC-3') (SEQ.ID. NO. 30) and #6617 (5'-AGCCTCGAGCTGGCCAGACACGGAACAAAT-3') (SEQ.ID.NO. 31) are used. The resulting DNA-fragment is 30 ligated to the T-overlaps of vector pDrive. The construct comprising mature vWF-cp is called pFP H251/8. For amplification of pre-prosequence of vWF-cp the primer

#66128 (5'-GGCGAATTCATGCACCAGCGTCACCCCG- 3') (SEQ. ID. NO. 32) and #6787 (5'-ACAGCATTAAACTAAGCCGCC-3') (SEQ.ID.NO. 33) are used. The resulting DNA-fragment is inserted into vector pDrive. The resulting construct is called pFP H262-35/14.

Example 7

Expression of vWF cleaving protease

10 The eukaryotic expression vector pcDNA3.1(+) (Invitrogen) is used for the expression of the full-length vWF cleaving protease (vWF-cp) under control of the human CMV promoter.

A 4.12 kB EcoRI/XhoI fragment is excised from the plasmid pFP H251/8, the fragment encompassing the cDNA sequence 15 encoding the mature vWF-cp from nucleotide nt 223 to 4284 (without pre-pro-sequence from nt 1 to nt 222) and inserted into the EcoRI/XhoI sites of pcDNA3.1(+). To complete the 5' -end of the cDNA to add the leader and propeptide sequence, a PCR is performed using plasmid pFP H262-35/14 20 which contains the cDNA sequence of vWF-cp from nt -10 (10 nt upstream from start codon ATG) to nt +824 in vector pDrive (Qiagen) as template and direct primer 6839 (5'- GATCGAATTCGCCGGCCACCATGCACCAGCGTCACCC CCG- 3') (SEQ.ID.NO. 34), harbouring the Kozak consensus sequence (underlined) 25 for the optimal context for the recognition of the start codon, and reverse primer 3113 (5'- CGGATAACAATTTCACACAGG- 3') (SEQ.ID.NO. 35), which binds in the lacZ region of pDrive vector. PCR is accomplished under standard reaction conditions using HotStar DNA polymerase (Qiagen) and Q- 30 solution (Qiagen). The amplified fragment comprising nt -10 to +824 is cut with EcoRI and AscI and used to replace the EcoRI/AscI fragment of the incomplete clone containing only

the mature sequence of vWF-cp. The latter construct encoding the complete sequence of vWF-cp called pCMV-vWFcp is further used for transfection and expression studies in mammalian cells.

5

For expression of vWF-cp, SKHep cells (ATCC) cells are transiently transfected with plasmid pCMV-vWFcp and, in parallel as a control with parental vector cDNA3.1(+), using Lipofectamine 2000 (Life Technologies, Inc).

10 Transfected cells are cultivated under standard conditions in DMEM/HAM's F12(1:1) medium (GIBCO) with 10% fetal calf serum, at a temperature of 37°C and 5% CO₂.

15 vWF-cp expression is analysed by Western blot, whereby samples of conditioned medium and cell harvest are subjected to SDS-PAGE on a 4%stacking/6% separation gel and Western-blot analysis is performed using anti-vWF-cp TTP patient plasma containing polyclonal anti-vWF-cp antibodies and goat-anti-hu-IgG (Fab-specific AB, SIGMA). VWF-cp specific bands are visualized by BCIP/NBT detection.

20 VWF-cp could be detected in sample of the cell lysate and seems to be mainly cell-associated. The results of the Westernblot analysis is shown in Figure 7, showing specific protein band of vWF-cp at about molecular weight of about 170kD reacting with patient sera.

25 The cell lysate of the vWF-cp expressing cells are further tested for vWF-cp activity according to the method as described by Furlan et al. (1996. Blood 87: 4235-4244) and vWF multimer analysis is performed in 1% SDS-agarose gel as described by Ruggeri et al. (1981. Blood 57: 1140-1143)

(Fig. 8). The biological activity of the vWF-cp expressed in mammalian cells could be clearly shown as high molecular weight vWF is degraded to vWF molecules having lower molecular weight (Fig. 8, lane 5).

5

Claims:

1. A vWF-cp polypeptide comprising (a) an amino acid sequence selected from the group of SEQ ID. NO 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4 and SEQ ID NO. 5, or (b) an amino acid sequence having at least 80% identity to the amino acid sequence of (a).
5
2. The polypeptide according to claim 1 encoding a polypeptide having an amino sequence as shown in Fig. 10 5 or a partial sequence of at least 12 amino acids thereof.
3. The polypeptide according to claim 1 or 2 having a vWF-cp activity.
15
4. The polypeptide according to any of the claims 1-3, wherein said polypeptide retains vWF cleaving protease activity in the presence of serine protease inhibitor and a calpain protease inhibitor.
20
5. An isolated polypeptide comprising an amino acid sequence having at least 80% identity to the amino acid sequence selected from the group of SEQ ID. NO 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4 and SEQ ID NO. 5.
25
6. An isolated polypeptide comprising an amino acid sequence selected from the group of SEQ ID. NO 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4 and SEQ ID NO. 5.
30

7. A substantially pure vWF -cp polypeptide selected from the group of a polypeptide comprising (a) an amino acid sequence selected from the group of SEQ ID. NO 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4 and SEQ ID NO. 5, or (b) an amino acid sequence having at least 80% identity to the amino acid sequence of (a).

5

8. A substantially pure vWF -cp polypeptide according to claim 7, wherein said polypeptide exhibiting an apparent molecular weight of about 180 KD, 170 kD, 160 kD or 120 KD in SDS-PAGE analysis under reducing conditions.

10

9. A substantially pure vWF -cp polypeptide according to claim 7, wherein said polypeptide exhibiting an apparent molecular wight of about 150 KD, 140 kD, 130 kD or 110 KD in SDS-PAGE analysis under non-reducing conditions.

15

20 10. A composition comprising a polypeptide according to any of the claims 1 - 9.

11. A composition according to claim 10 further comprising a divalent metal ion selected from the group of Ca^{2+} , Sr^{2+} , Ba^{2+} and Mg^{2+} .

25

30 12. A composition according to claim 10 or 11 comprising clusterin or an analog or derivative thereof having clusterin activity.

13. A nucleic acid molecule comprising a nucleic acid sequence encoding a vWF-cp polypeptide according to

any of the claims 1 to 7.

14. The nucleic acid molecule according to claim 13
5 encoding a vWF-cp polypeptide as shown in Fig. 5 or a
partial sequence thereof.

15. An expression vector comprising a nucleic acid
molecule according to claim 13.

10 16. A host cell comprising an expression vector
according to claim 15.

17. A method for production of a vWF-cp protease
polypeptide comprising the steps of

15 - growing a host cell according to claim 16 in a
nutrient medium under conditions to express said
polypeptide
- harvesting said polypeptide expressed and
- isolating said polypeptide.

20 18. A method of purification of vWF comprising the
steps of

25 - providing as a ligand a vWF-cp polypeptide according
to any of the claims 1 to 7,
- contacting a solution comprising vWF with said
polypeptide ligand under conditions whereby vWF is
bound to said ligand and
- recovering from said ligand purified vWF.

30 19. A method for production of anti-vWF cp
polypeptide antibodies wherein an animal is
immunized with a vWF-cp polypeptide according to any

of the claims 1 to 7 and anti-vWF-cp polypeptide antibodies are isolated from said animal.

20. A vWF-cp polypeptide binding molecule selected from
5 the group of antibodies, single chain antibodies, Fab-
or Fab'2-fragment, peptide or polypeptides with vWF
protease binding site, obtainable by a method wherein
a phage display library is screened for anti-vWF-cp
polypeptide binding molecule, the nucleic acid
10 sequence of positive clones are determined and a
nucleic acid molecule comprising the sequence is
cloned into an expression vector.

21. A method for purification of vWF -cp polypeptide
15 comprising the step of contacting a solution
comprising vWF -cp polypeptide with vWF protease
polypeptide binding molecule according to claim 20,
under conditions whereby vWF -cp polypeptide is bound
to said vWF -cp polypeptide binding molecule,
20 selectively eluting vWF -cp polypeptide from said
binding molecule and recovering purified vWF.

22. A method for detection of vWF -cp polypeptide in a
sample, wherein a solution suspected to contain vWF -
25 cp is contacted with a vWF-cp polypeptide binding
molecule according to claim 20, to allow the formation
of an vWF -cp polypeptide/ binding molecule complex
and detection said complex.

30 23. Use of a vWF-cp polypeptide according to any of the
claims 1 to 7 for the production of a preparation for
prophylaxis and therapy of thrombosis and

thromboembolic disease.

24. Use according to claim 23, wherein the
thromboembolic disease is thrombotic thrombocytic
5 purpura (TTP), Henoch-Schönlein purpura, preeclampsia,
neonatal thrombocytopenia or hemolyticuremic syndrome.

PURIFICATION SCHEME

1. Purification Step

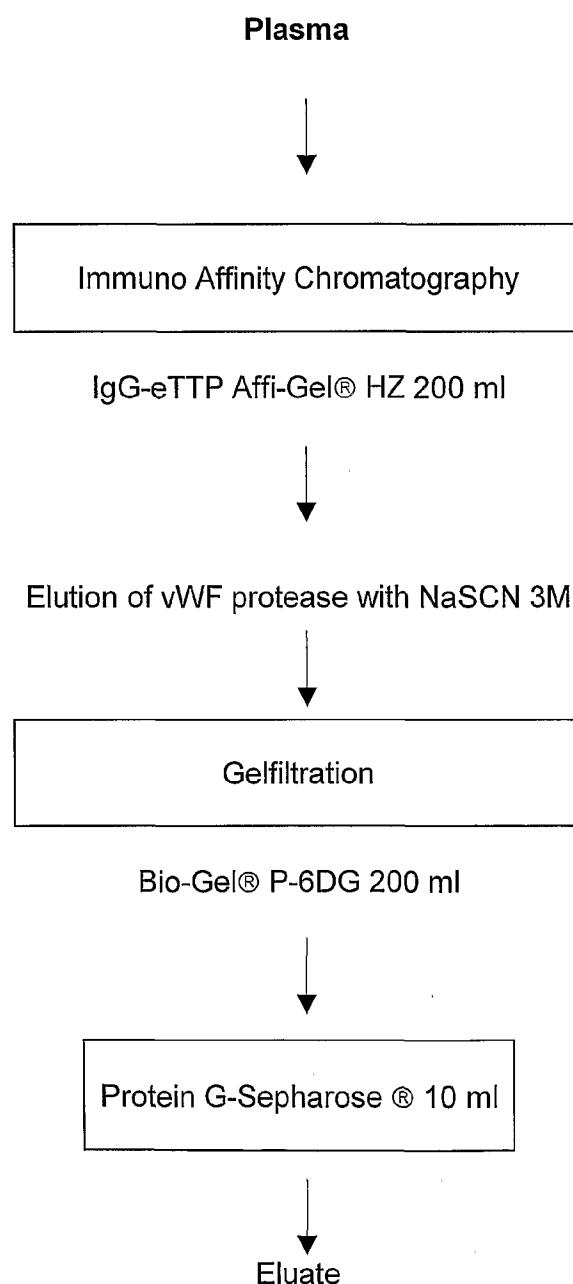


Figure 1

2. Purification Step

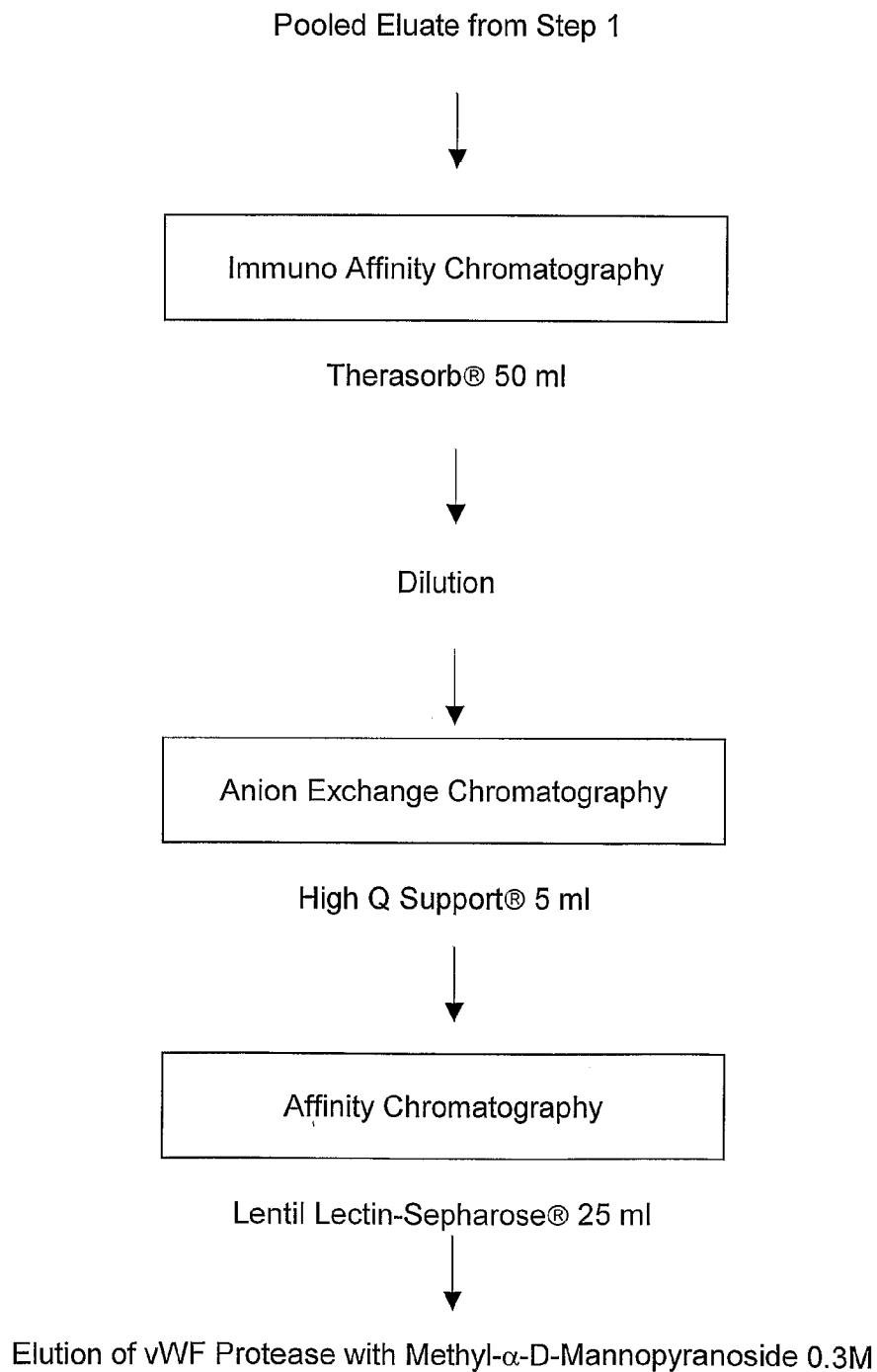


Figure 1

3. Purification Step

Pooled Eluate from Step 2



Affinity Chromatography

Heparin-Sepharose 5ml



Dialysis



Anion Exchange Chromatography

High Q Support® 5 ml



Gel Filtration

Sephacryl ® S-300 HR 500 ml



Elution of vWF Protease with NaCl 0.5 M

Figure 1

Optional: 4. Purification Step

Pooled Eluate from Step 3



Affinity Chromatography

Anti- α_2 -Macroglobulin-column 1ml



and/or optional



Affinity Chromatography

Anti-Clusterin column

Figure 1

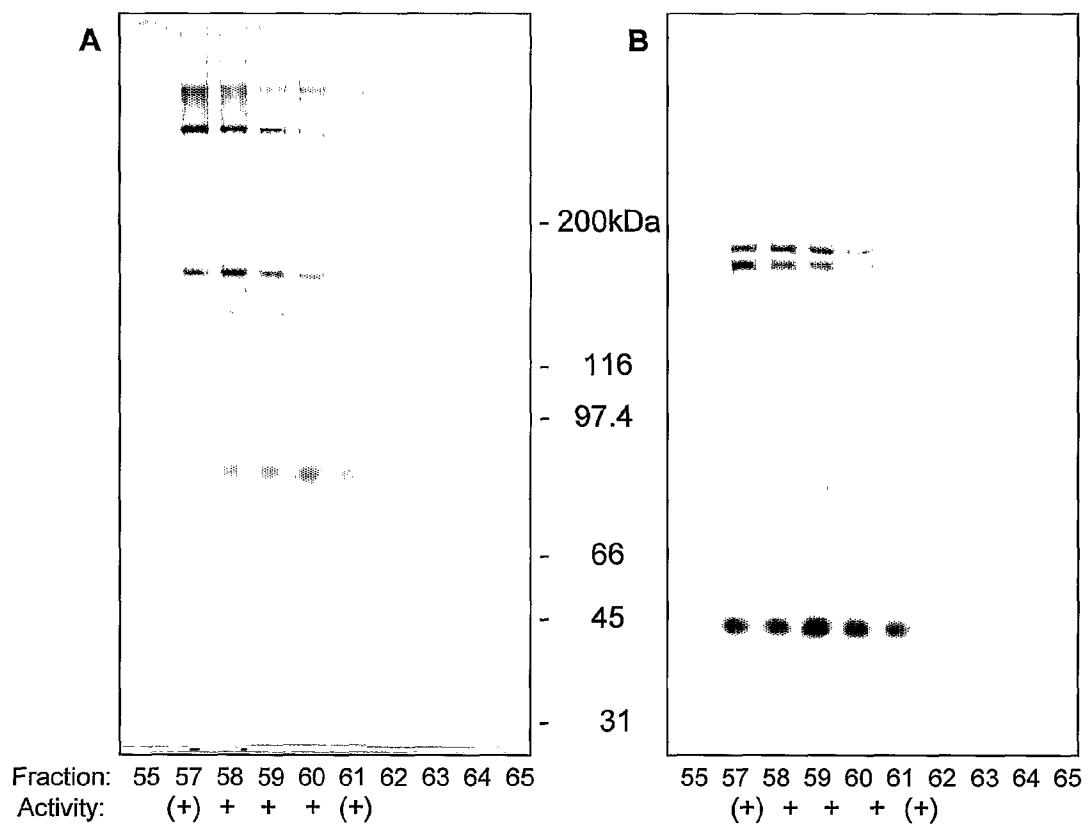


Figure 2

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Figure 3

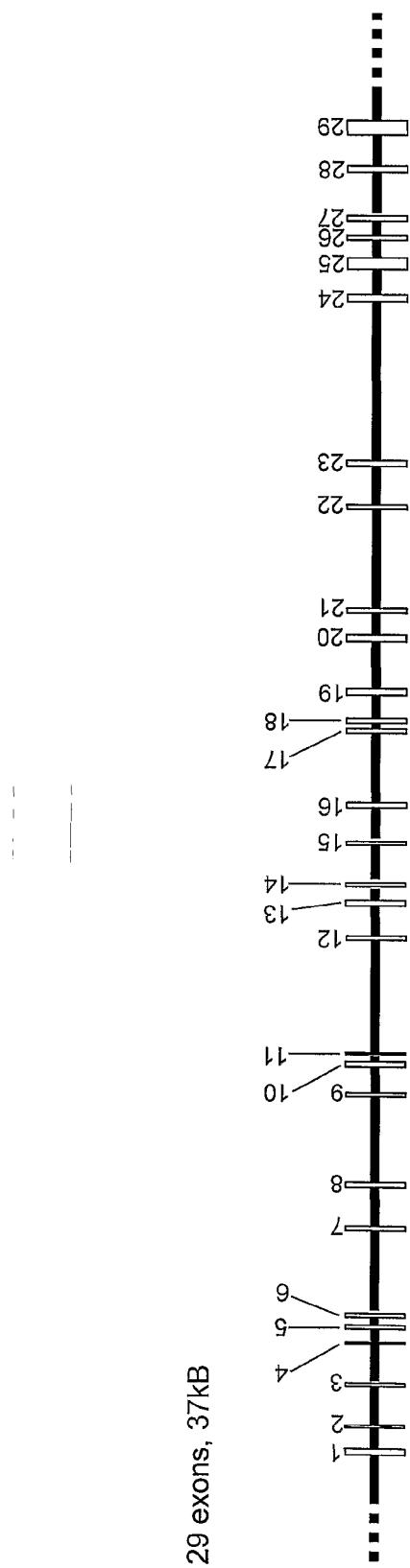


Figure 4

Figure 5A

Figure 5B

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Figure 5C

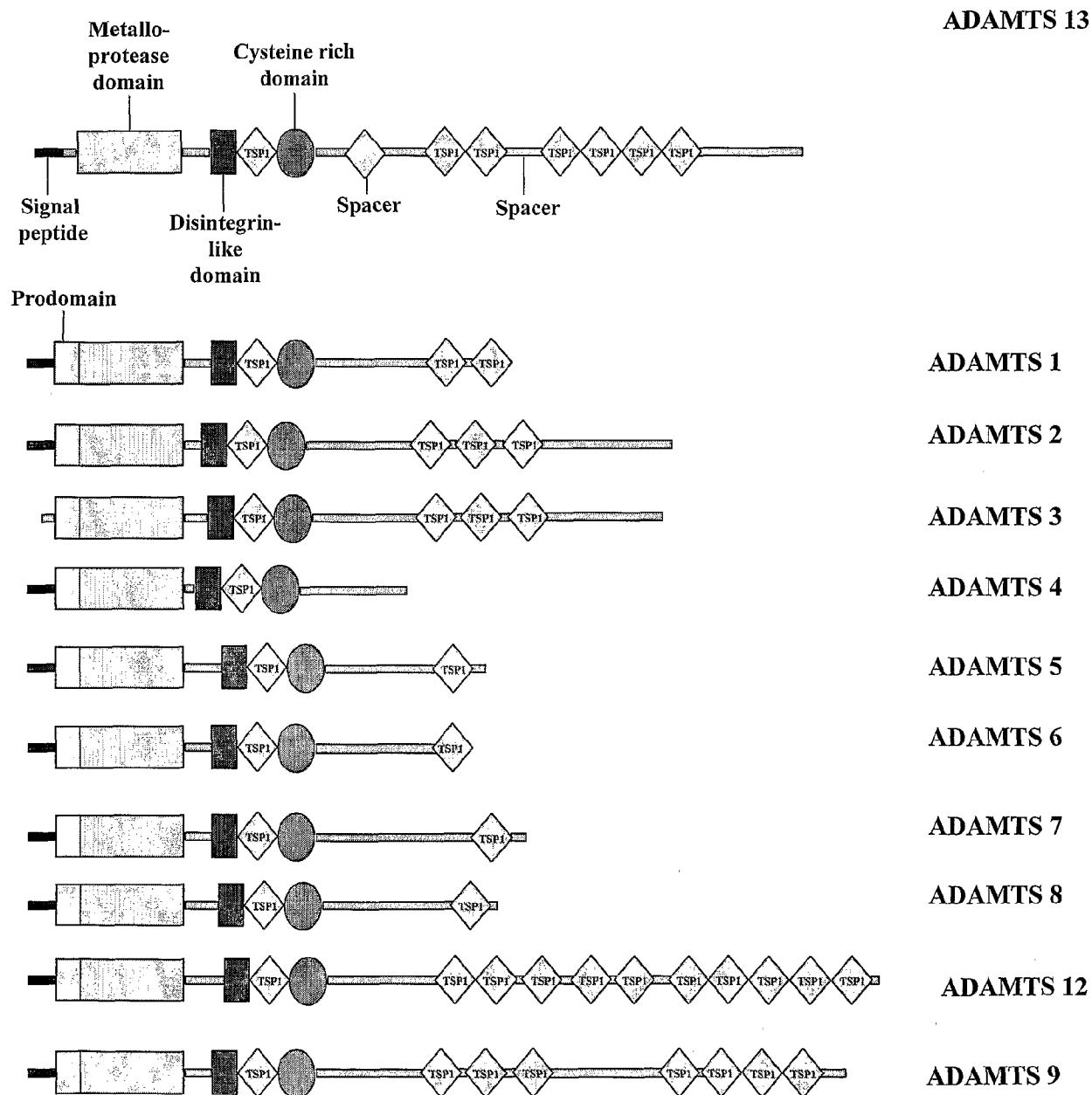
**Figure 6**



Figure 7



1 2 3 4 5

Figure 8

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25 tgg acc cct gcg gca ggg tcg tgc tcc gtc tcc tgc ggg cga ggt ctg Trp Thr Pro Ala Ala Gly Ser Cys Ser Val Ser Cys Gly Arg Gly Leu 900 905 910	2736
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15	15 ccc tcg ctg agt cca gcc acg agt aat gca ggg ggc tgc cgg ctc ttc	3984		
	Pro Ser Leu Ser Pro Ala Thr Ser Asn Ala Gly Gly Cys Arg Leu Phe			
	1315	1320	1325	
20	20 att aat gtg gct ccg cac gca cgg att gcc atc cat gcc ctg gcc acc	4032		
	Ile Asn Val Ala Pro His Ala Arg Ile Ala Ile His Ala Leu Ala Thr			
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	aac atg ggc gct ggg acc gag gga gcc aat gcc agc tac atc ttg atc	4080		
	Asn Met Gly Ala Gly Thr Glu Gly Ala Asn Ala Ser Tyr Ile Leu Ile			
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25	25 cgg gac acc cac agc ttg agg acc aca gcg ttc cat ggg cag cag gtg	4128		
	Arg Asp Thr His Ser Leu Arg Thr Thr Ala Phe His Gly Gln Gln Val			
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30	30 ctc tac tgg gag tca gag agc agc cag gct gag atg gag ttc agc gag	4176		
	Leu Tyr Trp Glu Ser Glu Ser Ser Gln Ala Glu Met Glu Phe Ser Glu			
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35	35 ggc ttc ctg aag gct cag gcc agc ctg cgg ggc cag tac tgg acc ctc	4224		
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40	40 caa tca tgg gta ccg gag atg cag gac cct cag tcc tgg aag gga aag	4272		
	Gln Ser Trp Val Pro Glu Met Gln Asp Pro Gln Ser Trp Lys Gly Lys			
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	Phe	Gln	Gln	Ser	Cys	Leu	Gln	Ala	Leu	Glu	Pro	Gln	Ala	Val	Ser	Ser
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5	Tyr	Leu	Ser	Pro	Gly	Ala	Pro	Leu	Lys	Gly	Arg	Pro	Pro	Ser	Pro	Gly
	50						55						60			
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	65						70						75			80
10	His	Leu	Glu	Leu	Leu	Val	Ala	Val	Gly	Pro	Asp	Val	Phe	Gln	Ala	His
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	Gln	Glu	Asp	Thr	Glu	Arg	Tyr	Val	Leu	Thr	Asn	Leu	Asn	Ile	Gly	Ala
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	Glu	Leu	Leu	Arg	Asp	Pro	Ser	Leu	Gly	Ala	Gln	Phe	Arg	Val	His	Leu
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	Cys His Thr Asp Pro Leu Asp Gln Ser Ser Cys Ser Arg Leu Leu Val			
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5	Pro Leu Leu Asp Gly Thr Glu Cys Gly Val Glu Lys Trp Cys Ser Lys			
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10	Gly Arg Cys Arg Ser Leu Val Glu Leu Thr Pro Ile Ala Ala Val His			
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15	Gly Arg Trp Ser Ser Trp Gly Pro Arg Ser Pro Cys Ser Arg Ser Cys			
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20	Gly Gly Gly Val Val Thr Arg Arg Arg Gln Cys Asn Asn Pro Arg Pro			
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	Ala Phe Gly Arg Ala Cys Val Gly Ala Asp Leu Gln Ala Glu Met			
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25	Cys Asn Thr Gln Ala Cys Glu Lys Thr Gln Leu Glu Phe Met Ser Gln			
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40	Thr Phe Gly Cys Asp Gly Arg Met Asp Ser Gln Gln Val Trp Asp Arg			
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	Thr Pro Asn Leu Thr Ser Val Tyr Ile Ala Asn His Arg Pro Leu Phe			
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50	Thr His Leu Ala Val Arg Ile Gly Gly Arg Tyr Val Val Ala Gly Lys			
	595	600	605	
	Met Ser Ile Ser Pro Asn Thr Thr Tyr Pro Ser Leu Leu Glu Asp Gly			
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Arg Val Glu Tyr Arg Val Ala Leu Thr Glu Asp Arg Leu Pro Arg Leu
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5 Glu Glu Ile Arg Ile Trp Gly Pro Leu Gln Glu Asp Ala Asp Ile Gln
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Val Tyr Arg Arg Tyr Gly Glu Glu Tyr Gly Asn Leu Thr Arg Pro Asp
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10 Ile Thr Phe Thr Tyr Phe Gln Pro Lys Pro Arg Gln Ala Trp Val Trp
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15 Trp Val Asn Tyr Ser Cys Leu Asp Gln Ala Arg Lys Glu Leu Val Glu
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20 Thr Val Gln Cys Gln Gly Ser Gln Gln Pro Pro Ala Trp Pro Glu Ala
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Cys Val Leu Glu Pro Cys Pro Pro Tyr Trp Ala Val Gly Asp Phe Gly
 740 745 750

25 Pro Cys Ser Ala Ser Cys Gly Gly Leu Arg Glu Arg Pro Val Arg
 755 760 765

Cys Val Glu Ala Gln Gly Ser Leu Leu Lys Thr Leu Pro Pro Ala Arg
 770 775 780

30 Cys Arg Ala Gly Ala Gln Gln Pro Ala Val Ala Leu Glu Thr Cys Asn
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35 Pro Gln Pro Cys Pro Ala Arg Trp Glu Val Ser Glu Pro Ser Ser Cys
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40 Pro Gly Ala Asp Gly Leu Glu Ala Pro Val Thr Glu Gly Pro Gly Ser
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Val Asp Glu Lys Leu Pro Ala Pro Glu Pro Cys Val Gly Met Ser Cys
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45 Pro Pro Gly Trp Gly His Leu Asp Ala Thr Ser Ala Gly Glu Lys Ala
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Trp Thr Pro Ala Ala Gly Ser Cys Ser Val Ser Cys Gly Arg Gly Leu
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 15 Leu Glu Pro Cys Pro Pro Arg Trp Lys Val Met Ser Leu Gly Pro Cys
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 30 Gly Asp Gly Ile Gln Arg Arg Asp Thr Cys Leu Gly Pro Gln Ala
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5 Thr Trp Arg Lys Met Cys Arg Lys Leu Leu Asp Met Thr Phe Ser Ser
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10 Gly Val Leu Leu Arg Tyr Gly Ser Gln Leu Ala Pro Glu Thr Phe Tyr
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15 Pro Ser Leu Ser Pro Ala Thr Ser Asn Ala Gly Gly Cys Arg Leu Phe
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50 <400> 35
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